SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

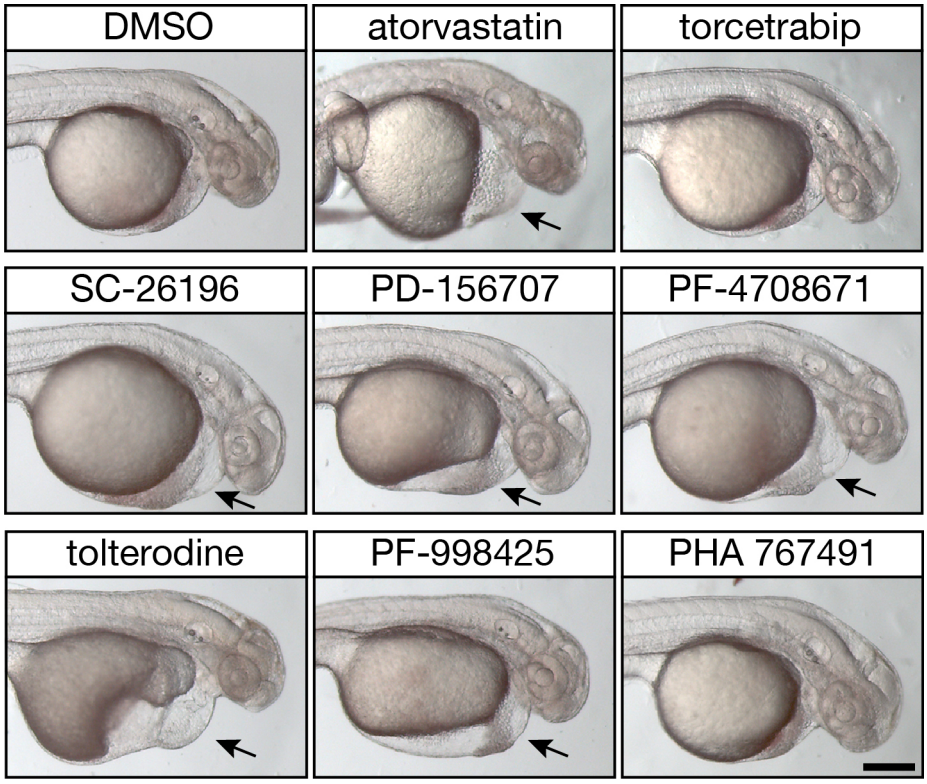

Figure S1: Phenotype of zebrafish embryos treated with 8 arrhythmogenic drugs.
Live images of vehicle (DMSO) or drug-treated embryos at 48 hpf. Zebrafish were allowed to develop until tailbud stage and immersed in either vehicle (1% DMSO in egg water) or 50 µM compound in egg water, both containing PTU to prevent pigmentation. At 48 hpf embryos were analyzed.
Arrows indicate pericardiac and inflow tract edema. Scale bar: 200 µM.

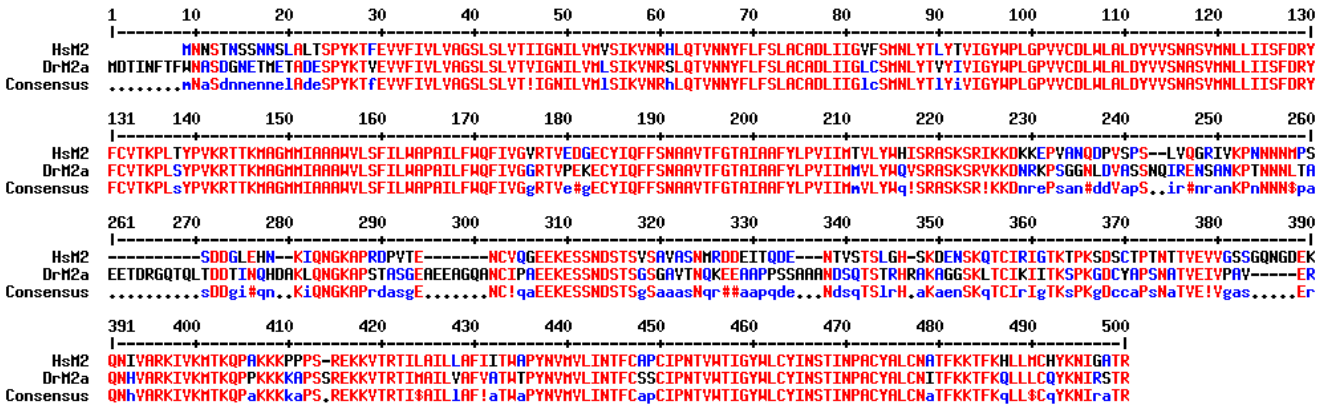

Figure S2: Amino acid alignment of human muscarinic receptor M2 and zebrafish M2a Red indicates identical amino acids and blue shows similar residues. HsM2, Homo sapiens M2 receptor; DrM2a, Danio rerio muscarinic receptor 2a.

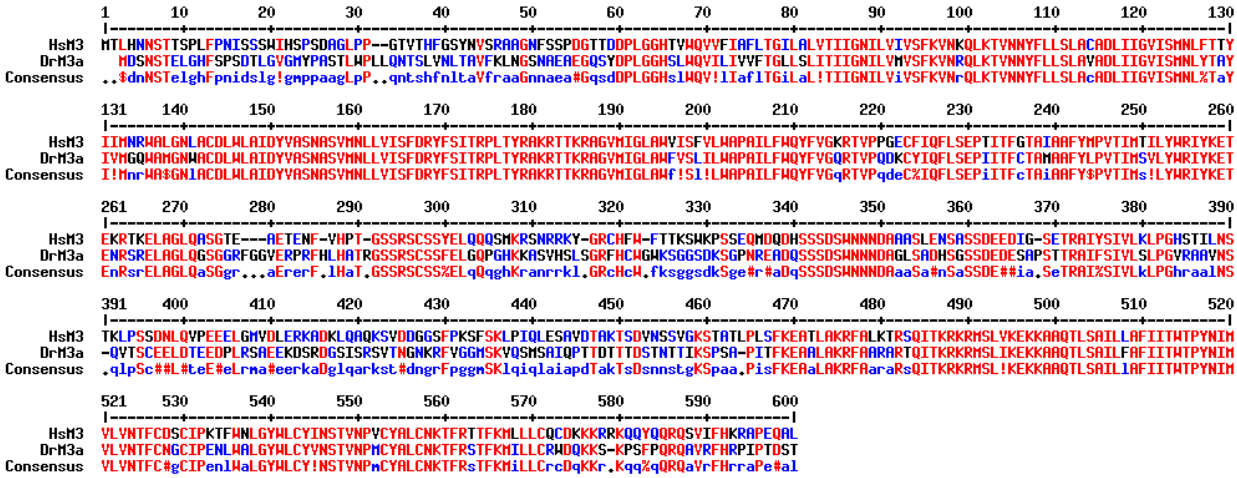

Figure S3: Amino acid alignment of human muscarinic receptor M3 and zebrafish M3a Red indicates identical amino acids and blue shows similar residues. HsM3, Homo sapiens M3 receptor; DrM3a, Danio rerio muscarinic receptor 3a.

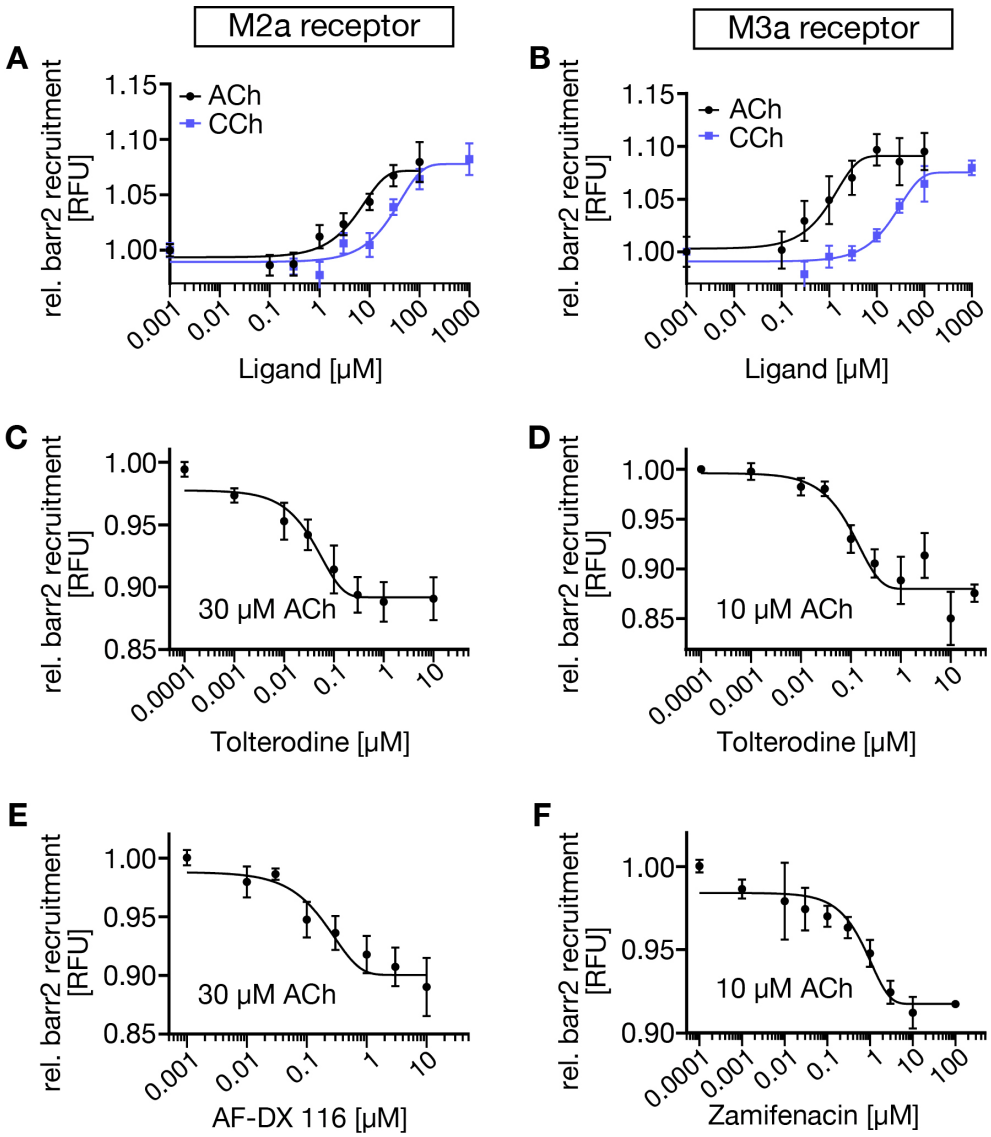

Figure S4: BRET assay to test functionality of zebrafish muscarinic receptors
A, Zebrafish M2a receptors can be stimulated by acetyl choline (ACh) as well as carbachol (CCh) as measured by relative (rel.) beta-arrestin 2 (barr2) recruitment.
B, Both, acetyl choline as well as carbachol function on zebrafish muscarinic M3a receptors as full agonists.
C and **D**, Tolterodine functions as an antagonist on both, M2a and M3a receptors. ACh concentrations were chosen to achieve full receptor activation prior to tolterodine administration.
E, The M2-specific antagonist AF DX-116 has antagonistic functions on zebrafish M2a receptors.
F, Zamifenacine efficiently inhibits beta-arrestin 2 recruitment upon stimulation with ACh in M3a expressing cells.

RFU, relative fluorescent units. Graphs show mean ± SD of two measurements performed in duplicate.

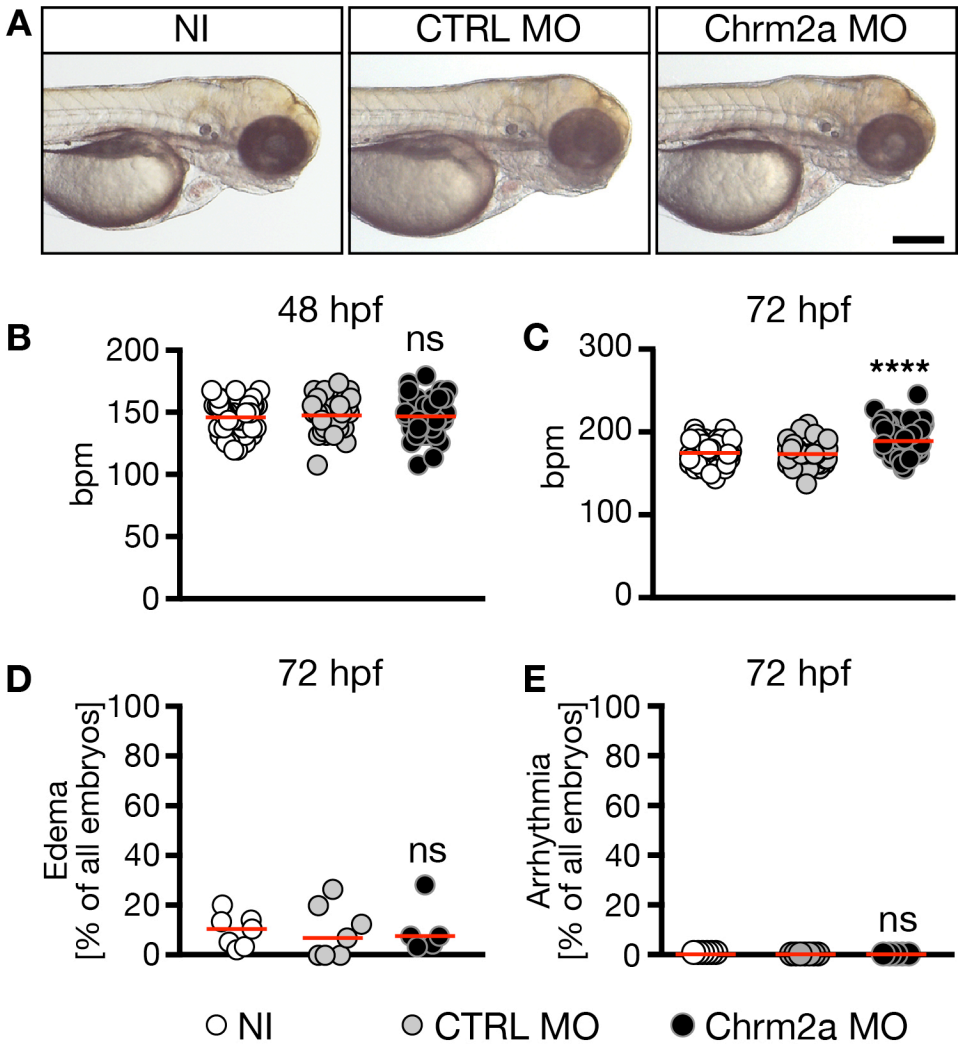

Figure S5: Depletion of an M2 muscarinic receptor in zebrafish embryos does not mimic the tolterodine phenotype

A, Live images of wild-type embryos (NI), control-injected (CTRL MO) embryos and those injected with a translation blocking MO against the muscarinic M2a receptor (Chrm2a MO) at 72 hpf. Scale bar: 200 µm.

B, Knockdown of M2a receptors does not change the heart rate at 48 hpf. n= 4 experiments with 57 embryos per condition. Circles indicate individual embryos.

C, M2a receptor-depleted embryos develop tachycardia at 72 hpf. n= 4 experiments with 55 embryos per condition. Data analyzed by Kriuskal-Wallis test with **** indicating $p<0.001$. Circles indicate individual embryos.

D, Knockdown of M2a receptors does not induce edema formation. n= 6 experiments with 146-148 embryos per condition. One circle per experiment.

E, Loss of M2a receptor does not produce arrhythmia. n= 6 experiments with 146-148 embryos per condition. One circle per experiment.

Red lines indicates median. All data analyzed by Kruskal-Wallis tests.

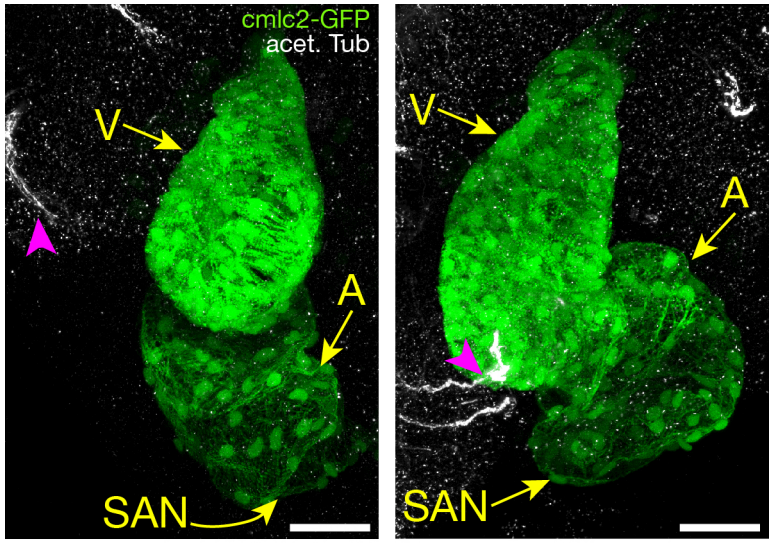

Figure S6: Innervation of zebrafish heart at 48 hpf
Confocal images of two different 48 hpf zebrafish hearts from cmcl2-GFP transgenic fish immunostained for acetylated tubulin (white) to visualize neurons. Note that the axons (magenta arrowheads) have not reached the SAN, yet. A, atrium, V, ventricle, SAN, sinoatrial node. Scale bar: 50 µm.

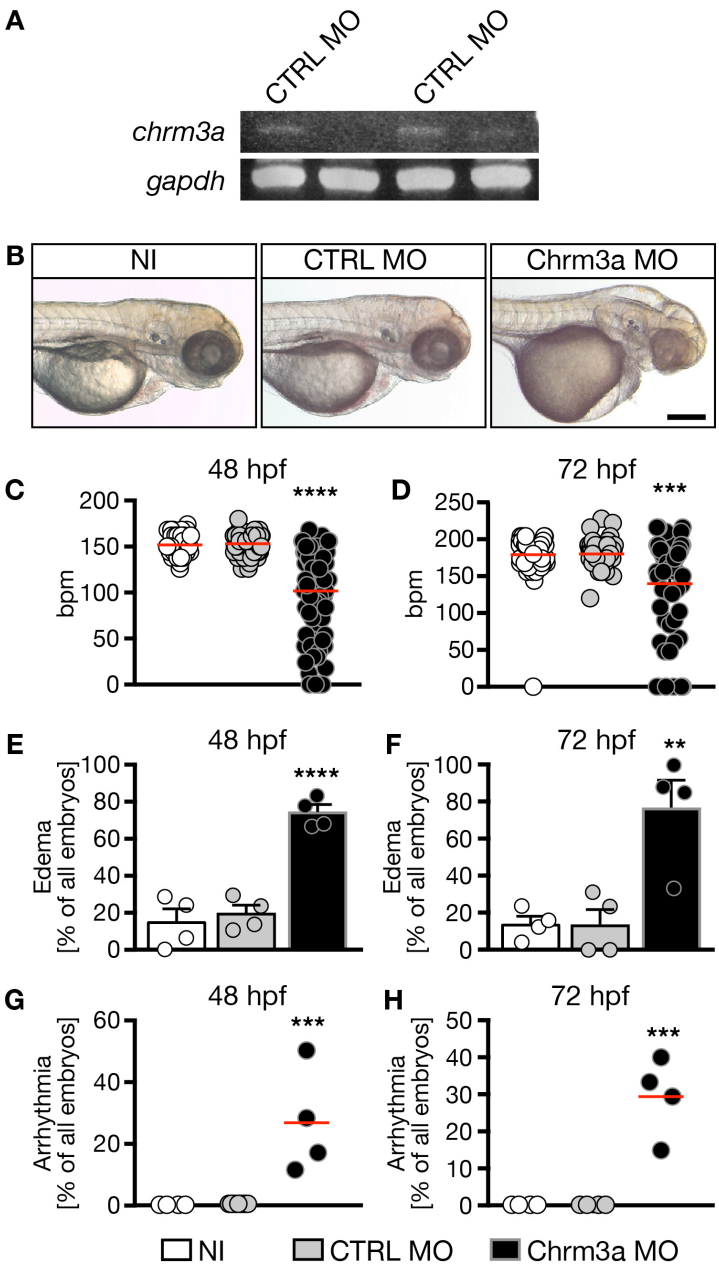

Figure S7: M3a muscarinic receptor depleted zebrafish embryos show impaired cardiac function

A, Injection of a splice blocking MO against the muscarinic M3a receptor (Chrm3a) results in splicing defects. Image shows RT-PCR of control (CTRL MO) or Chrm3a MO injected zebrafish at 24 hpf. In the presence of Chrm3a MO a weaker band for *chrm3a* could be detected. As control *gapdh* was used.

B, Live images of wild-type embryos (NI), control-injected (CTRL MO) embryos, and embryos injected with a splice blocking MO against the muscarinic M3a receptor (Chrm3a MO) at 72 hpf. Scale bar: 200 µm.

C, Knockdown of M3a receptors causes bradycardia with a highly variable heart rate at 48 hpf. n= 5 experiments with 74-75 embryos per condition. Kruskal-Wallis test with **** indicating p<0.0001.

D, At 72 hpf, M3a receptor-depleted embryos still display a highly variable heart rate. n= 5 experiments with 59-74 embryos per condition. Kruskal-Wallis test with *** indicating p=0.003.

E, At 48 hpf, M3a receptor morphant embryos have developed pericardiac and inflow tract edema. n= 4 experiments with 76-87 embryos per condition. Data analyzed by One-way ANOVA with **** indicating p<0.0001.

F, Most M3a depleted embryos maintain pericardiac edema at 72 hpf. n= 4 experiments with 76-87 embryos per condition. Data analyzed by One-way ANOVA with ** indicating p=0.0032

G, M3a receptor depleted embryos display arrhythmia at 48 hpf. n= 4 experiments with 76-87 embryos in per condition. Kruskal-Wallis test with *** indicating p=0.0002.

H, Arrhythmia in 72 hpf embryos injected as indicated. n= 4 experiments with 76-87 embryos per condition. Kruskal-Wallis test with *** indicating p=0.0002.

C and **D**: each symbol represents one embryo. Red line: median.
E and **F**: Data summarized in bar graphs show mean ± SEM.
G and **H**: Each symbol represents one experiment. Red line: median.

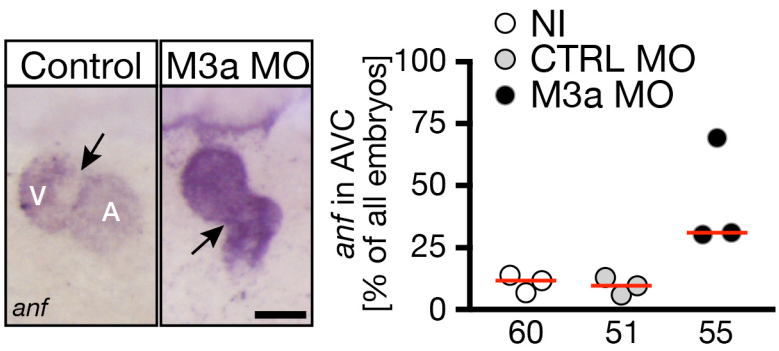

Figure S8: Chrm3a knockdown in zebrafish embryos promotes *anf* expression in the AVC
MO-mediated Chrm3a loss-of-function results in *anf* expression in the non-contractile myocardium of the AVC. Left panel shows representative images of in situ hybridizations at 48 hpf. Graph summarizes three experiments with 51 to 60 embryos in total. Red line indicates median. * $p<0.0507$, Kruskal-Wallis-test.

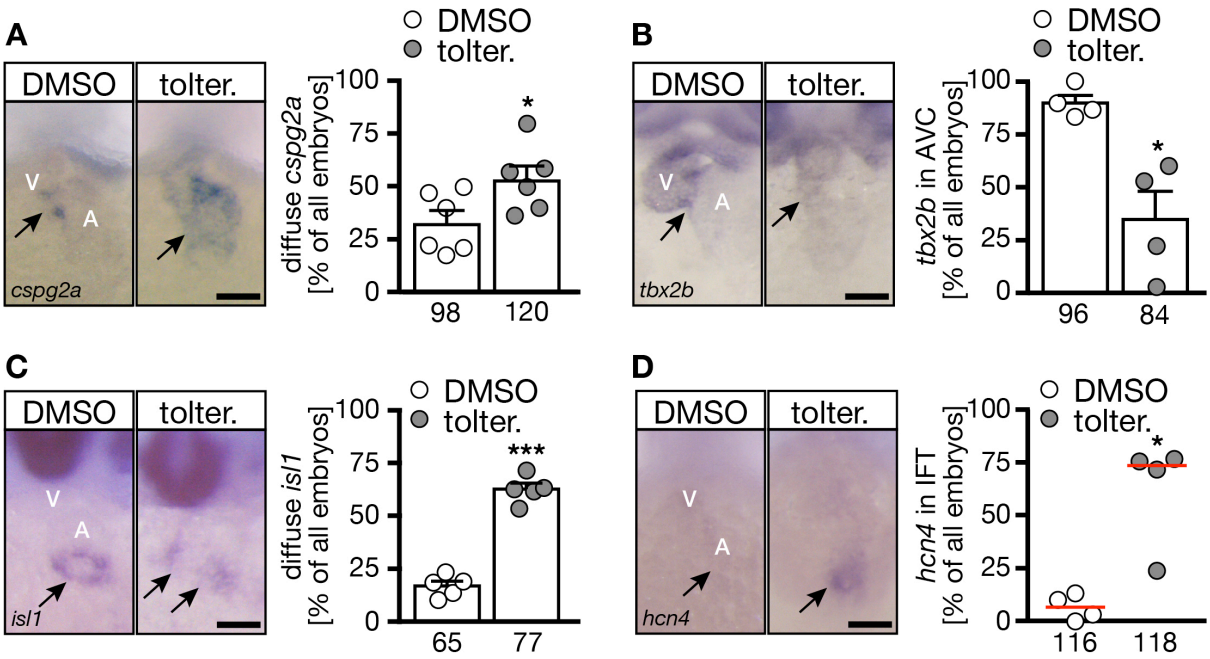

Figure S9: Tolterodine promotes pacemaker cell fate at the expense of AVC cells in zebrafish embryos

A-D, Representative images of whole mount in situ hybridizations at 48 hpf. A, atrium; V, ventricle. Number of embryos are indicated in the bars. n=4-6 experiments. Bar graphs show mean±SEM. Scale bars: 50 µm.

A, *cspg2a*, which is normally restricted to the AVC, is expressed throughout the heart upon tolterodine treatment. * p=0.0384, two-tailed t-test with Welch's correction.

B, *tbx2B* expression is lost upon tolterodine treatment. * p=0.0216, two-tailed t-test with Welch's correction.

C, Tolterodine provokes a less confined expression of the pacemaker marker *isl1*. *** p<0.0001, two-tailed t-test with Welch's correction.

D, Tolterodine increases *hcn4* expression in the area of the inflow tract. * p=0.0286, two-tailed Mann-Whitney test.

Graphs display mean ± SEM and or median (red, **D**).

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